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# Research Article
# Immuno Modulatory Effects of Bergapten Attenuates D-galactose-induced Aging Model in Balb/C Mice

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## Abstract

**Background and Objective:** Aging is featured by many mechanisms of protective immunity exhibit defects. Bergapten (BG), is a furanocoumarin derived from herbal and citrus extracts can act as antioxidant and selective anticancer agents. However, its immune regulation properties have not been well documented. The current study aimed to investigate whether bergapten would attenuate immuno senescence and to explore its immuno modulatory effect on immune responses in D-galactose-induced aging Balb/C mice. **Materials and Methods:** In the beginning of the experiment, survival test was performed on infected aging Institute of Cancer Research (ICR) mice with bergapten treatment. Male Balb/C mice were separated into normal or D-galactose-induced aging model. Production of serum cytokines such as anti-oxidant enzyme and malondialdehyde (MDA) were determined. Then, the model group was separated into three subgroups that were treated with BG and normal diet for 14 days by oral gavage, respectively. Namely groups D-gal+BG (20, 100 mg $kg^{-1}$) groups and D-gal group. Flow cytometric analysis and enzyme activity assays were used in isolated splenocytes. Modulatory effects of bergapten on T cell proliferation and the production of IFN-γ and IL-4 were also investigated. **Results:** BG (20 or 100 mg $kg^{-1}$) group reverses body weight and spleen index in aging progress significantly. Further experimental results showed that BG (20 mg $kg^{-1}$) treatment not only promoted T cell proliferation but also up-regulated IFN-γ and IL-4cytokinesin aging mice. Moreover, BG (20 mg $kg^{-1}$) treatment enhanced Th and Tc responses, which may participate in the process of eliminating the virus in an old age. **Conclusion:** This *in vivo* study firstly shown that bergapten has immuno modulate effect against D-galactose induces aging Balb/C mice.

Key words: Bergapten, immuno modulatory effect, D-galactose, immuno senescence, lymphocyte proliferation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

Human life span has been expected that the elderly (over 65 year) population increase over 800 million by the year 2025[1]. It is well reported that age causes immune deficiency in the adaptive immune system[2] and a loss of proper immune regulation are more likely to be shown in an aged innate immunesystem[3,4]. Previous studies demonstrated that a model of natural aging effect in various tissues of rodents can be induced by subcutaneous injection of D-galactose[5]. Therefore, D-galactose-induced aging can be a suitable model for age-related research.

It is well known that aging is a complex process, which can lead to immune deficiency and deeply worsen the immune system. Immune deficiency with age is featured in increased rate of morbidity and other chronic diseases, including infectious diseases. Overproduction of MDA, various antioxidant enzymes like SOD and TAC have been demonstrated play a critical role in age-related changes. Apart from those serum parameters, a consistently observed changes during aging occurred in naïve T cells. Since aged animals and elderly individuals have decreased T cell proliferation, subset of $CD8^+$ T cell responses specific to influenza virus are diminished with aging in both mice and humans[6,7]. However, aged $CD4^+$ T cells suppress activated microglia and pathogenic T cells, which contribute significantly to the impaired humoral response[8]. In addition, old mice had an impaired response to infection such as declined inflammatory cytokine IFN-γ, as well as the Th2 cytokine IL-4.

Bergapten (5-Methoxypsoralen, BG), chemical structure shown in Fig. 1a cumarine-derivate compound from virous medicinal plants. In southern China, BG is one of main components from thee thanol extract of *Ficus hirta Vahl*. root (a traditional material for food and medicine by Hakka people in Guangdong province). It has been shown to be antiproliferative, anti cancer and has been used in skin photo chemotherapy for decades[9-11]. Biological functions such as antidepressant[12], antioxidative[13-15] and anti-inflammatory[15] have been well documented in previous studies. In addition, suppression of TNF-α and IL-6 release on lipopolysaccharide challenge has been shown by bergapten administration[15,16], the molecular mechanism underlying interactions of bergapten with immune responses to age-related changes have not been clarified yet. In this study, bergapten was applied to treat mouse models of aging-induced immune deficiency, based on the optimal proportional allocation. The immuno modulatory effect of bergapten was investigated.

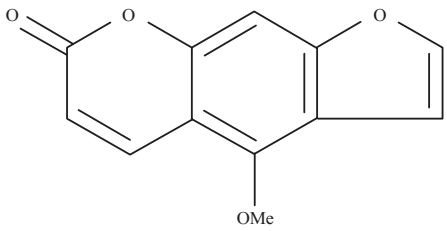


Fig. 1: Chemical structure of bergapten

## MATERIALS AND METHODS

The entire study was conducted from 2016.06.01 to 2017.03.31.

**Chemical compound and other reagents:** Bergapten (BG) or 5-methoxypsoralen ($C_{12}H_8O_4$,> 98%, CAS number 484-20-8), was obtained from Sigma-Chemical (Milan, Italy). D-galactose was purchased from Sinopharm Chemical Reagent (Zhangjiang, SH, China). Fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicillin and streptomycin were acquired from Millipore (Bedford, MA, USA). Dulbecco's modified eagle medium (DMEM) and RPMI-1640 medium, were obtained from Gibco (NY, USA). Dimethyl sulfoxide, Superoxide dismutase (SOD), malondialdehyde (MDA), enzyme linked immuno spot kits such as interferon-γ (IFN-γ) and interleukin-4 (IL-4) and total antioxidant capacity (TAC) were obtained from Jiancheng bio engineering institute (Nanjing, China). Anti-CD3, anti-CD4 and anti-CD8 were obtained from BioLegend (CA, USA).

**Experiment mice:** Institute of Cancer Research (ICR) mice and Balb/C (male)mice were bought from the Laboratory Animal Center of Guangdong Province (Foshan, China). Ethical approval number was SCXK2015-0003. Balb/c mice aged 8-12 weeks. All experiments and the methods used in the study were conducted according to the America National Institutes of Health guide and the *in vivo* study approved by the Animal Ethics Committee of Guangdong Medical University. Mice were kept under air conditioning in plastic cages with room temperature of 25°C, 12 h light/dark cycle daily. At least 1 week needed for animals to acclimatize difference environment before the experiment. Laboratory mice daily food was also purchased from the Laboratory Animal Center of Guangdong Province. And mice were provided food and water freely throughout the experiment study *ad libitum*.

**Survival test in ICR aging mice with bergapten:** The concentration of 180 mg $kg^{-1}$ $day^{-1}$ D-galactose was used to

establish the mimetic aging effect in mice. A total of 60 ICR aging mice weighing between 23 and 25 g were divided into four experimental groups (15 mice per group) in randomly. H1N1 influenza virus was obtained from Shenzhen Disease Control and Prevention Center (Shenzhen, GD, China). To examine the effects of bergapten on the life span of virus infected aging mice, vehicle or BG diets were given to virus infected aging mice. Normal diet was given to aging mice that is control group. The studies were conducted on the basis of the guidelines set by the NIH (7th Edition, USA). Before being fed orally to mice, bergapten was suspended in 1% Tween 80 solution. Lethal dose ($LD_{50}$) titer of H1N1 influenza virus was 100 plaque-forming units. After anesthetized by pentobarbital, three groups of infection mice were established by intranasal inoculation. The concentration of virus was approximately $LD_{50} \times 2$. The day of virus inoculation was defined as day 0. Two groups of these infection mice were administered bergapten at single doses of 20, 100 mg $kg^{-1}$ $day^{-1}$. Another group of infection mice (Virus group) were treated with distilled water as drinking water. The animals were monitored daily for survival and changes in general behavior. On the 14th day, the mice were sacrificed under anesthesia and vital organs (heart, kidneys, lung, spleen and liver) were removed for macroscopic examination. The anti-infection effect of bergapten on aging mice was assessed on the basis of survival rate[17-19].

**Treatment schedule of Balb/C mice with bergapten:** Firstly, mice were given D-galactose (180 mg $kg^{-1}$) subcutaneous injections for 30 days. To evaluate the establishment of the aging-related effect in mice, serum samples of Balb/C mice were collected from tail vein. Those collected samples were used for the measurement of SOD, MDA and TAC. Kits for measurement of SOD, MDA and TAC were following the manufacturer's protocols. In the second part, BG was suspended in 1% Tween 80, so that have different concentrations of 20 and 100 mg $kg^{-1}$. Balb/C aging mice were administrated at 20 and 100 mg $kg^{-1}$ body weight daily with the suspensions. Aging Balb/C mice were freely divided into three groups: negative control group received 1% Tween 80 solution only, named D-gal group. Positive groups were received BG administration at the dose of 20 and 100 mg $kg^{-1}$, named D-gal+BG(20) group and D-gal+BG (100) group, respectively. All aging animals were received gavage in a dose of BG or vehicle once daily, normal group received regular diet with vehicle as saline group (n = 6 in each group). On the 14th day of treatment, mice were sacrificed by chloral hydrate, blood collected into serum-separating tubes and centrifuged at 1000 g (4°C, 20 min) to isolate serum. Blood and serum were immediately stored at -80°C until biochemical determinations were performed[17]. The equation for spleen index was shown below:

$$\text{Spleen index (\%)} = \frac{\text{Spleen weight}}{\text{Body weight}} \times 100$$

**Preparation of mononuclear cell suspension:** Following the dissection of the abdomens, spleen was removed aseptically from the aging mice and spleens from Balb/C mice were gently meshed by a stainless steel sieve. Red blood cells were lysed in 0.85% $NH_4$-Tris-HCl buffer and then the splenocytes were washed twice. Splenic mononuclear cells, were isolated by laboratory centrifugation and counted using a microscope (×100). Try pan blue exclusion test was conducted to determine the cell viability (>95%). After that, splenic mono nuclear cells were cultured in RPMI-1640 medium, which added with 10% FBS, 50 IU $mL^{-1}$ penicillin and 50 μg $mL^{-1}$ streptomycin. Effects of bergapten on T lymphocyte proliferation and flow cytometry were assessed by using the splenic cell suspension.

**Detection of T lymphocyte proliferation in Balb/C mice:** Spleen cells of experiment mice were harvested. In this part, lymphocyte proliferation was measured by methyl thiazolyl tetrazolium assay. Briefly, spleen cells ($2.5 \times 10^6$ cells $well^{-1}$) were incubated with concanavalin A (Con A, 5 μg $mL^{-1}$) in a 96-well culture plates wells. Total volume of 200 mL in each well. All test wells were carried out in triplicate. After adding 20 μL of the methyl thiazolyl tetrazolium solution (concentration of 5 mg $mL^{-1}$) in the last 4 h of incubation. The plates were centrifuged at 1000 g. And then carefully discarded the supernatant, dimethyl sulfoxide (100 μL) was added in each well. Finally, T cell proliferation was calculated as the optical density (OD) values. Plates were read at 570 nm by enzyme-linked immuno sorbent micro plate reader RT-6000, which obtained from Leidu Co., Ltd (Shenzhen, China).

**Cytokine detection assay:** Enzyme linked immuno spot kits were used to quantitatively determine IFN-γ and IL-4 levels of the isolated serum. Detection of IFN-γ and IL-4 were conducted following the manufacturers' instructions. Spots were scanned and calculated by iSpot reader System, which obtained from AID Co., Ltd (Strassberg, Germany).

**Flow cytometry analysis:** Spleen-cell suspension were prepared at a density of $10^7$ $mL^{-1}$ to flow cytometry analysis. The cells were incubated at 4°C in 30 min. Then washed in the fluorescence activated cell sorter (FACS) buffer (0.05% PBS, 2 mmol $L^{-1}$ FCS, EDTA, 0.01% $NaN_3$) and incubate for 30 min on ice. Immuno phenotype was determined by using mixture of anti bodies including anti-CD3, anti-CD4 and anti-CD8. After extensive washing, the cells were resuspended in 400 µL DAPI, analyzed cellsona Calibur™ flow cytometer, which obtained from BD Biosciences (CA, USA).

**Statistical methods:** Results are described as Mean±standard (Mean±SD). Survival curves was analyzed using the Kaplan-Meier method[20]. The statistical significance set as $p<0.05$. Group comparisons were analyzed by one-way analysis of variance (ANOVA). GraphPad Prism 6.0 projects (CA, USA) have been used for drawing figures.

## RESULTS

**Effect of bergapten on mortality:** Survival rate of mice after 14 days post-bergapten administration was shown in Fig. 2. From the survival test, the virus group survival rate was only 38.69%, while 92.31 and 64.29% did in the BG treated groups (20 and 100 mg $kg^{-1}$). Compared with the virus group, both groups of BG treatment significantly decreased the mortality rate. These results have shown a protective effect of BG against the virus-induced death and at a lower dose of BG treatment would increase the survival rate of virus-infected mice more significantly.

**Production of SOD, TAC and MDA in the serum of Balb/C mice:** After 30 days of subcutaneous injections, mice treated with D-galactose have a significant difference with normal group. It is well documented that the activity of the anti-oxidant enzymes decreased in the aging process, such as SOD and TAC. As shown in Table 1, the baseline of TAC in the normal group was 1.04±0.07 mmol $L^{-1}$, while the TAC of D-galactose group was 0.42±0.09 mmol $L^{-1}$ ($p<0.01$). Contrary to the previous two parameters, the value of MDA in normal group was 1.01±0.17 µmol $g^{-1}$ and this value was increased in D-galactose group, which was 5.62±0.63 µmol $g^{-1}$. Notably, the ability of oxidation seemed to increase in mice after subcutaneous injection of D-galactose (180 mg $kg^{-1}$ $day^{-1}$).

**Effect of bergapten on physiological parameters:** Body weight and spleen index were calculated as physiological

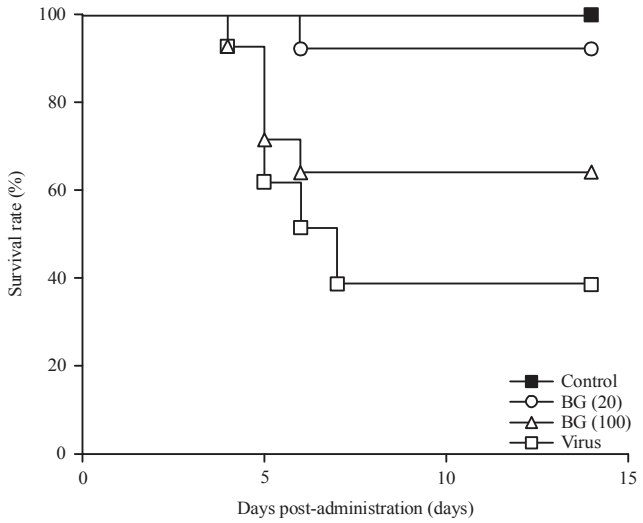


Fig. 2: Effect of bergapten on survival rate of infected aging mice after post-bergapten administration
Time course of survival of each mouse was recorded until the 14th day after viral infection (n = 15)

Table 1: Production of SOD, TAC and MDA in the serum of Balb/C mice. The activity of SOD and TAC in serum were determined using a kit. The content of MDA in serum was determined using a kit, n = 6

| Groups | SOD (U $mg^{-1}$) | TAC (mmol $L^{-1}$) | MDA (µmol $g^{-1}$) |
|---|---|---|---|
| Normal | 77.17±6.18 | 1.04±0.07 | 1.01±0.17 |
| D-galactose | 47.02±12.29## | 0.42±0.09## | 5.62±0.63## |

Significant differences are compared to the normal group at ##$p<0.01$, values are the Mean±SD

parameters of immune deficiency caused by aging progress. As shown in Fig. 3a, at the beginning of the experiment, no difference in mean body weight of different groups was noticed. On the last day (14th day), compared with D-gal group, BG (20 and 100 mg $kg^{-1}$) treatment significantly alleviated body weight changes ($p<0.01$) associated with aging progress. Spleen weights were recorded to calculate the spleen index. As shown in Fig. 3b, compared with D-gal group, two doses of BG (20 and 100 mg $kg^{-1}$) had shown protective effect on spleen index. Moreover, the aging mice treated with a lower-dose of BG diet were shown a higher spleen index ($p<0.01$). These results indicate the positive effects of the BG treatment on the immune system, which may, at least partially, explain the anti-infected effects in aging mice.

**Promotion of T lymphocyte proliferation by bergapten:** T cell population was closely related to the life span of individual. Appropriate T cell population in the periphery is highly protect age-related shift towards memory and senescent. For further investigating the effect of BG diets on aging-induced immune deficiency, the researchers measured

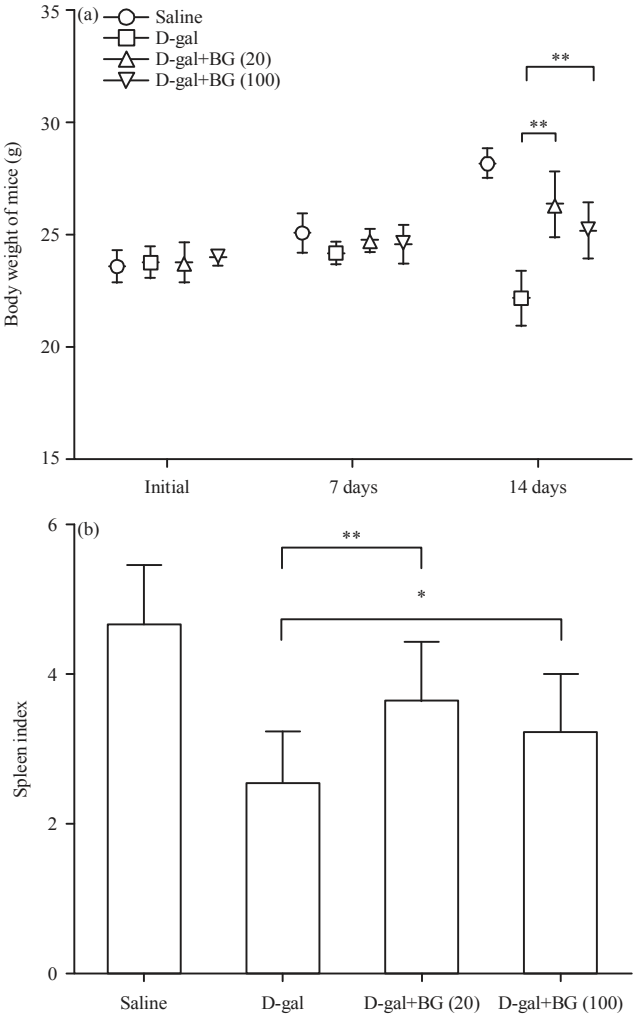


Fig. 3(a-b): Effect of bergapten on physiological parameters, (a) Body weight weighted on the day of 1st, 7th and 14th, n = 6 and (b) Spleen index was calculated in every Balb/C mice and then, Mean±SD was described in each group, n = 6
Significant differences are compared to the D-gal group at *p<0.05 and **p<0.01, values are the Mean±SD

T cell population in the spleen after feeding the mice BG diets for 14 days. T cell proliferation was calculated as the OD values.

As shown in Fig. 4, the bergapten diet increased the relative T cell population in D-galactose-induced aging mice. D-gal+BG (20 or 100) group was higher in T cell population response to under conditions of non-drug support aging mice ($p<0.01$ or $p<0.05$, respectively), thus, BG diet has a notable enhancement of the T lymphocyte proliferation. The results suggested that BG diet reverses T cell population in aging status.

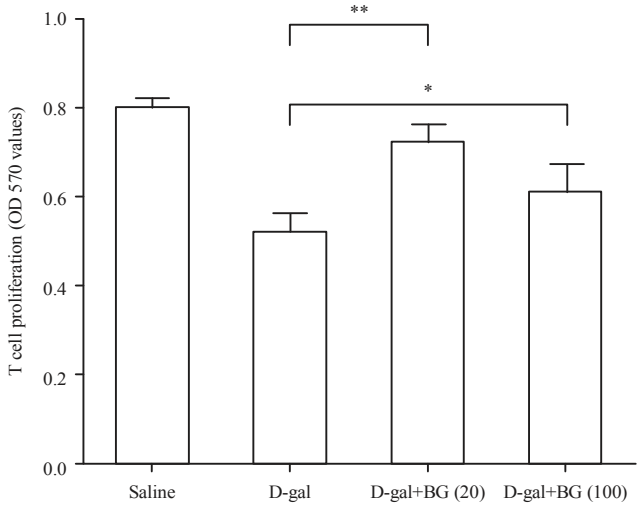


Fig. 4: Promotion of T lymphocyte proliferation by bergapten. Aging mice were divided into three groups with BG (20 mg $kg^{-1}$), BG (100 mg $kg^{-1}$) and saline alone, respectively. The spleen cells of these mice were harvested and cultured in RPMI-1640. Con A (5 μg $mL^{-1}$) was used for non-specific stimulation. After stimulation, OD values were measured, n = 6
Significant differences are compared to the D-gal group at *p<0.05 and **p<0.01, values are the Mean±SD

**Immuno modulatory effects of bergapten in Th1/Th2 cytokines:** T-helper 1 (Th1) and T helper 2 (Th2) cells played vital roles in immune system. The production of IFN-γ and IL-4 are Th1- and Th2- related cytokines response to foreign antigens. To further evaluate the immuno modulatory effects of bergapten, spleen cells from aging mice were separated. Concentrations of IFN-γ and IL-4 were assessed by the isolated cells. As shown in Fig. 5, the BG (20 mg $kg^{-1}$) diet significantly increased the secretion of IFN-γ and IL-4 from D-galactose-induced aging mice ($p<0.05$ and $p<0.01$, respectively). Moreover, BG (20 mg $kg^{-1}$) treatment was more likely to increase the secretion of IL-4. It was known that IL-4 was the typical cytokine in Th2 immune response. Hence, these data suggested that bergapten (20 mg $kg^{-1}$) diet had immuno modulatory effect on immune responses, with a predominance of Th2 responses.

**Enhancement of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells in aging mice by bergapten:** To make further investigation of BG effect on the activation of T cells, the proliferation of double positive cells of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ were measured by fluorescence activated cell sorter (FACS). Percentage of these T cells had been shown in Fig. 6. Compare to the saline group, the production of $CD3^+$ $CD4^+$ and $CD3^+$

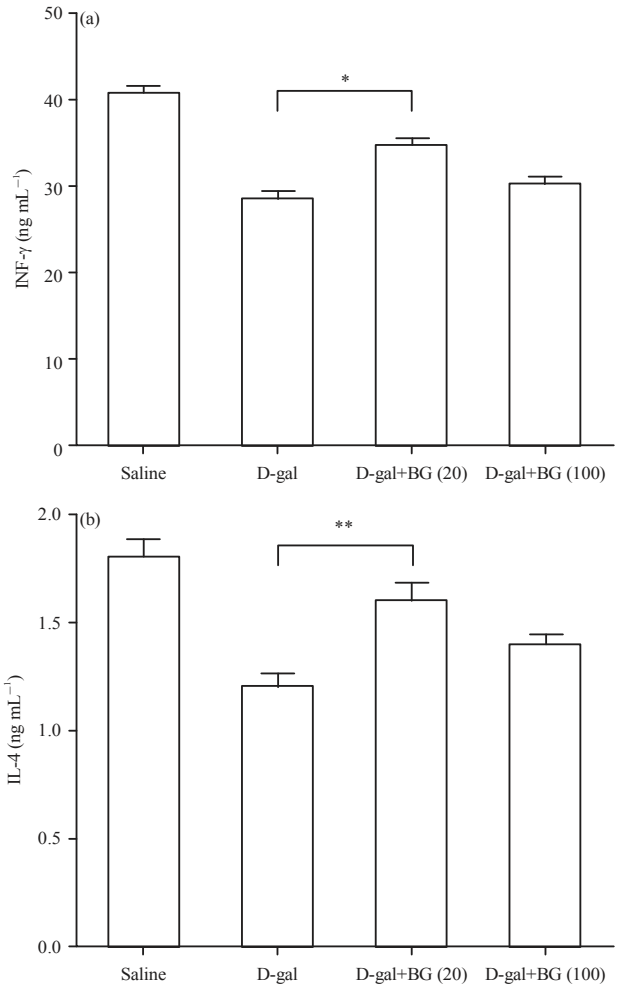


Fig. 5(a-b): Immuno modulatory effects of bergapten in Th1/Th2 cytokines. Cells were plated at a density of $1\times10^6$ cell per well. (a) IFN-γ and (b) IL-4 production by splenocytes in response to stimulation was measured by enzyme-linked immunospot (ELISPOT) assay. Each column represents the Mean±SD of spots scanned and calculated by iSpot reader system, n = 6

Significant differences are compared to the D-gal group at *p<0.05 and **p<0.01, values are the Mean±SD

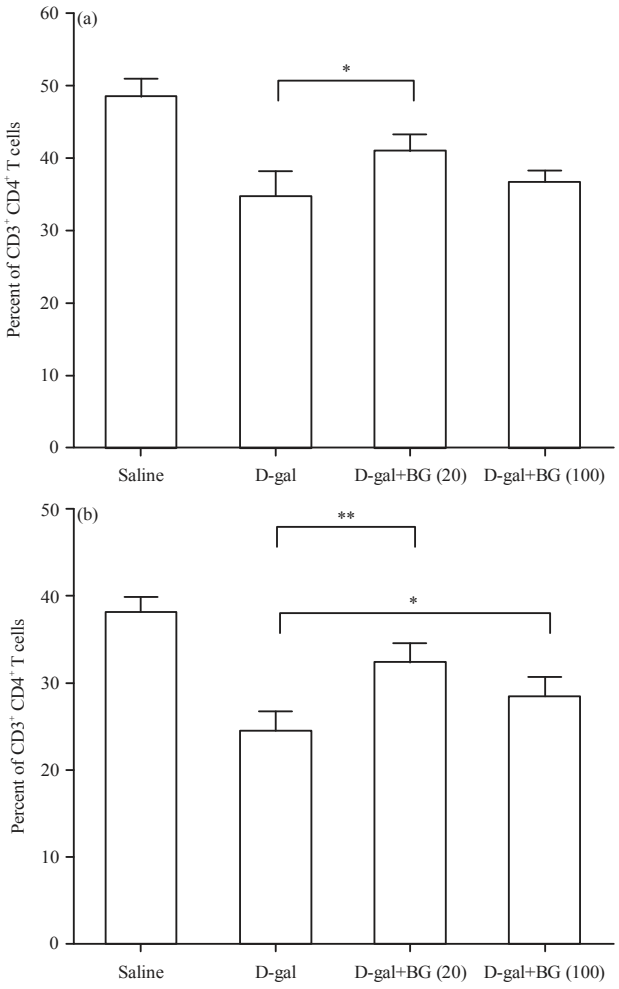


Fig. 6(a-b): Enhancement of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in aging mice by bergapten. Cells were stained with FITC-labeled anti-CD3 mAb, PE-labeled anti-CD4 mAb or PE-labeled anti-CD8 mAb (b) and analyzed on a Calibur™ flow cytometer. The percentage of (a) $CD3^+$ $CD4^+$T cells and (b) $CD3^+$ $CD8^+$ T cells in the lymphocyte population was calculated from representative experiments, n = 3

Significant differences are compared to the D-gal group at *p<0.05, **p<0.01, values are the Mean±SD

$CD8^+$ T cells inhibited extremely in non-drug support D-gal group. Statistical analysis between D-gal group and saline group were not shown in this figure. D-gal+BG (20) group displayed a significantly up-regulation in $CD3^+CD4^+$ T cells, when compared with that of detected in D-gal group ($p<0.05$). However, compared to the D-gal group, BG (100 mg $kg^{-1}$) treatment had no significant difference with the percent of $CD3^+$ $CD4^+$ T cells. On the other side, BG up-regulated the expression of $CD3^+$ $CD8^+$ T cells and significant differences were detected between the D-gal+BG (100) group and D-gal group ($p<0.05$). Similarly, compared with the D-gal group, D-gal+BG (20) group significantly up-regulated the expression of $CD3^+$ $CD8^+$ T cells ($p<0.01$). Thus, BG (20 mg $kg^{-1}$) was more likely restore antigen-specific $CD4^+$ and $CD8^+$ T cells in aging models, which may help to curing chronic infections.

## DISCUSSION

This study illustrated that BG treatment could increase survival rate of virus-infected mice, regulate the poor antioxidant status and attenuated aging-induced immune deficiency via enhanced Th and Tc responses in the D-galactose induced aging model.

In the last decade, immunomodulatory effects of natural compounds have been reported in many studies[21,22]. Molecular mechanisms of bergapten in immunological function have been studied in bone-destructive diseases and human breast cancer cells[23,10]. Learning from previous reports on the mechanisms of immunosenescence[24], it hypothesized that BG may rescue immune senescence by modulating immune response.

D-galactose-induced aging mice as a research model for investigating the immunological function of BG. In the present study, aging-induced immune deficiency model was verified by performing survival test and the assessment of TAC in serum. It was well documented that the aging process in older individuals were distinguished with altered immune system function and an increased risk of infection[25]. In view of intrinsic changes to the peripheral environment with aging, the elderly individual is more likely to have poor antioxidant status, which further impair immune system function[26]. Serum parameter of TAC was clearly associated with aging process from newly reported studies[27-29]. The result of TAC in this study also confirmed that aging mice were featured in poor antioxidant status.

In adaptive or specific immunity, a variety of macro phages and different types of T cells, are the primary mediators in immune system against aging induced diseases[30,31]. Enzyme-linked immunospot assay and flow cytometry analysis were conducted to investigate whether bergapten could rescue immune deficiency on the activation of T cells. Th1 type cells secrete its typical cytokine IFN-γ, as a protective function in activation of native T cells[10]. Th2 cytokines produce cytokines such as IL-4 were responsible for chronic infections[32]. Results from present study shown that BG (20 mg $kg^{-1}$) diet significantly increased the secretion of IFN-γ and IL-4 from D-galactose-induced aging mice. And the results supported that BG could modulate immunity against aging-induced immune deficiency by activating T cell response. The study next assessed whether BG could also modulate cellular immune functions in the elderly.

A blunted T-cell proliferation is a hallmark of mammal immunosenescence. Previous study has demonstrated that $CD8^+$ and $CD4^+$ T cells responses are defective in older mice[33]. Hence, clear insights into the regulation of $CD8^+$ and $CD4^+$ T cells is of great needed in immunotherapeutic strategie[34]. According to the results, after being treated with BG (20 mg $kg^{-1}$), the proliferations of T lymphocyte were significantly higher compared with that of in D-gal group (Fig. 4). Both $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ responses were elicited after being treated with BG (20 mg $kg^{-1}$) in aging mice (Fig. 6). BG plays a role in preservation of antigen-specific $CD4^+$ and $CD8^+$ T cells in aging models. The result indicated that treatment with bergapten (20 mg $kg^{-1}$) increases both Th and cytotoxic T (Tc) cells and that could provide effective protection against aging-induced immune deficiency. It provides strong evidence that cellular immunity had been significantly enhanced after being treated with BG (20 mg $kg^{-1}$) in aging mice. In addition, it is well known that $CD4^+$ and $CD8^+$ T cells possess unique ability to eliminate virus-infected cells. Enhanced Th and Tc responses activate different effector cells to clear the virus infection. Thus, BG (20 mg $kg^{-1}$) treatment may participate in the process of eliminating the virus in an old age. However, the study was failed to address a key target of BG action in T cells for the control of survival and immune response.

## CONCLUSION

Together the results presented in this study indicated that BG therapy can modulate immunity against viral epidemics and attenuate aging-induced immune deficiency. These effects may be attributed to the activation of the Th and Tc responses in D-galactose induced aging Balb/C mice.

## SIGNIFICANCE STATEMENTS

This study provides information on the pharmaco dynamics of BG. It would be helpful for researcher to uncover the immunomodulatory effect of BG that many researchers were not able to explore. Thus, a new theory on BG as potential therapeutic immunomodulatory agent may be arrived at.

## ACKNOWLEDGMENTS

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